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THE EFFICACY OF PHYSIOLOGICAL AND PHARMACOLOGICAL N-METHYL-D-ASPARTATE RECEPTOR BLOCK IS GREATLY REDUCED UNDER HYPERBARIC CONDITIONS

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Abstract—Human divers exposed to hyperbaric pressure may suffer from cognitive and motor impairments thought to be related to high pressure effects per ce. These effects, termed high pressure neurological syndrome (HPNS), appear at pressure above 1.1 MPa. HPNS involves CNS hyperexcitability that is partially attributed to augmented responses of the glutamatergic N-methyl-D-aspartate receptor (NMDAR). NMDAR is blocked by Mg^{2+} (physiologically) and by DL-2-Amino-5-phosphonopentanoic acid (AP5, pharmacologically). We have recently reported that hyperbaric pressure augments rat hippocampus NMDAR synaptic response and generates hyperexcitability. We now test pressure effects on the blockade efficacy of $Mg^{2+}$and AP5. Under high pressure conditions more than double $[Mg^{2+}]_o$ and $[AP5]_o$ were needed to achieve similar effects on NMDAR synaptic response's amplitude, decay time, and time integral comparable to control conditions. $[Mg^{2+}]_o$ and $[AP5]_o$ concentration-response curves and the concentration for 50% responses' inhibition (IC_{50}s) showed similar normalized pattern at control and pressure for each parameter. We conclude that hyperbaric pressure reduces the efficacy of these NMDAR blockers that may be associated with the receptor conformational change(s). This provides additional mechanism for pressure over activation of NMDAR. Taken together with our previous reports, high pressure modification of NMDAR activity significantly contributes to CNS hyperexcitability and possibly for long term vulnerability. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: hippocampus, magnesium, AP5, NMDAR, HPNS, synaptic transmission.

High ambient (e.g. hydrostatic) pressure above 1.1 MPa induces the high pressure neurological syndrome (HPNS) in animals and human divers (for review: Bennett and Rostain, 2003). HPNS is conceivably associated with CNS hyperexcitability. It is mainly characterized by dizziness, nausea, stomach cramps, vomiting, muscle twitching, and tremors (Brauer, 1984), EEG changes and a reduction in cognitive function (Abraini, 1997). At greater depths, myoclonia (Darbin et al., 2000), convulsions and seizures may occur (Bennett and McLeod, 1984). Rats seem to be more resistant to high pressure than humans. Tremor and convulsions thresholds for rats are at 6 MPa and at 9 MPa respectively (Brauer et al., 1974).

Previous neuropharmacological studies at high pressure indirectly showed that there may be an increase of excitatory N-methyl-D-aspartate receptor (NMDAR) response in CA1 pyramidal cells (Fagni et al., 1985, 1987b) and a possible decrease in inhibitory GABA activity. These findings may explain the hyperexcitability observed in rat hippocampal slices *in vitro* (Zinebi et al., 1988, 1990) and the epileptiform activity in the whole animal (Fagni et al., 1985).

By using microdialysis and electrochemical techniques in freely moving rats, Darbin et al. (1999, 2000, 2001) and Kriem et al. (1999) showed an increased sensitivity of NMDAR and an elevated extracellular concentration of dopamine and glutamate at 8 MPa. The microdialysis technique was performed at the level of the basal ganglia and has been implicated in locomotor and motor hyperactivity.

However, the authors report that the increase of glutamate level recorded in the striatum with helium pressure might have an exocytotic and a non-exocytotic origins. Earlier studies on invertebrates have shown that high pressure depressed neuromuscular glutamatergic excitatory synapses by decreasing the quantal content but not the quantal size (Golan et al., 1995, 1996). Grossman and Kendig (1988) showed that both excitatory (glutamatergic) and inhibitory (GABAergic) synapses in crustacean neuromuscular synapses were depressed at pressure. Furthermore, Fagni et al. (1987a); Etzion and Grossman (2000) and Talpalar and Grossman (2003) have also shown that high pressure depressed single normal glutamatergic synaptic responses in brain slices from hippocampus, cerebellum, and dentate gyrus respectively. Reduction in presynaptic Ca^{2+} entry was also indirectly implicated with this synaptic depression.

Despite the above reported general depression of evoked glutamate synaptic release, we have recently demonstrated in CA1 hippocampal brain slices (Mor and Grossman, 2006) that, the single isolated NMDAR field excitatory post-synaptic potential (fEPSP) is augmented at 10.1 MPa. This unusual augmentation includes increased initial slope, larger amplitude and longer decay time. Furthermore, in the same preparation, population spikes (PSs) generated by NMDAR fEPSP tended to appear earlier in a train indicating hyperexcitability. However, the

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Abbreviations: AP5, DL-2-amino-5-phosphonopentanoic acid; DNQX, 6,7–dinitroquinoxaline -2,3-dione; fEPSP, field excitatory post-synaptic potential; HPNS, high pressure neurological syndrome; IC_{50}, antagonist's concentration needed to reach fifty per cent response inhibition; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; PS, population spike.

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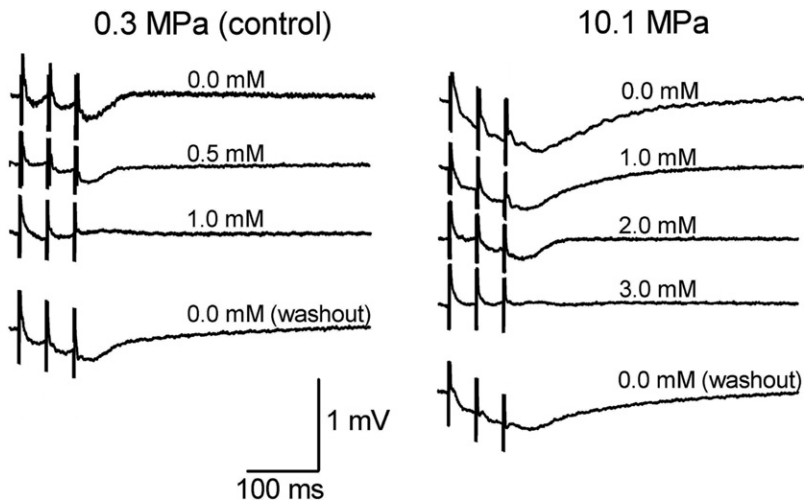

Fig. 1. $[Mg^{2+}]_o$ blockade of NMDAR fEPSP. Single experiment (last 3 of a 5 stimuli train are shown, 25 Hz) demonstrating increased blockade of the synaptic response by elevating $[Mg^{2+}]_o$. Under high pressure conditions (10.1 MPa) complete block of the fEPSPs was achieved by 3 mM $[Mg^{2+}]_o$, three times higher than the control conditions. Twenty min washout of the $[Mg^{2+}]_o$ resulted in almost full recovery.

trains' PSs number, amplitude, and frequency were decreased indicating hypo-excitability (Mor and Grossman, 2007).

Up to date, it seems that NMDAR can be significantly affected by high pressure causing fEPSP augmentation and PSs firing pattern change. Recently NMDAR ionic currents have been measured directly under high pressure. Preliminary experiments of expressing NMDAR in *Xenopus laevis* oocytes showed that one of the most abundant NMDAR subtypes (in the mammalian brain—NR1-1a+NR2A) activation is augmented under hyperbaric conditions (Mor et al., 2008).

NMDAR is both a ligand and voltage gated non specific cation channel (Cull-Candy et al., 2001). Mg^{2+} physiologically blocks the channel pore and is removed by depolarization. Reducing the normal extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$, ~1 mM) greatly potentiates NMDAR function (Mayer et al., 1984; Nowak et al., 1984). Therefore, our previous measurements of NMDAR fEPSPs (Mor and Grossman, 2006, 2007) were made under $[Mg^{2+}]_o=0$, in order to allow maximal response in the absence of AMPA receptors depolarization. DL-2-Amino-5-phosphonopentanoic acid (AP5) is a potent NMDAR pharmacological inhibitor. It is a competitive antagonist on the NMDAR glutamate binding site (Clements and Westbrook, 1994; Laube et al., 1997; Cull-Candy et al., 2001).

Despite the reduction in evoked glutamate release in the hippocampal formation at pressure, NMDAR activity is potentiated. Up to date, NMDAR blockade mechanisms have never been studied at high pressure. Therefore, the major goal of the present study was to examine pressure effects on the efficacy of NMDAR blockade by $[Mg^{2+}]_o$ and $[AP5]_o$ in an attempt to understand the pressure induced augmentation in NMDAR response.

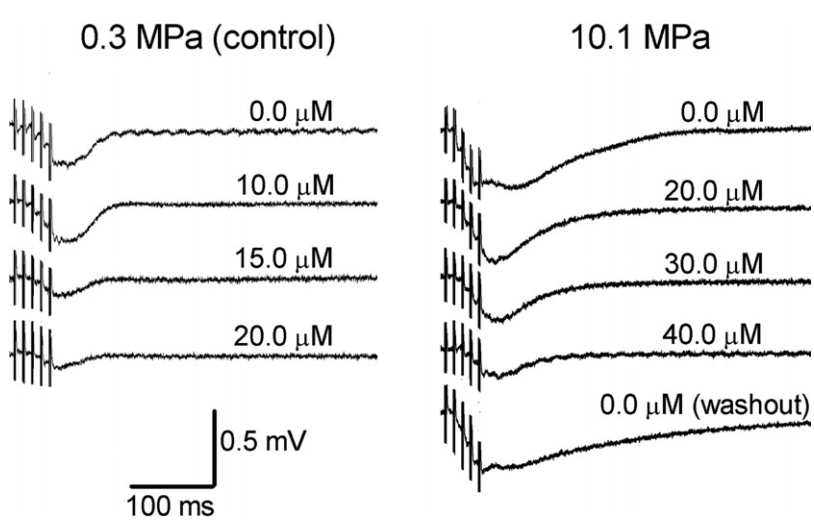

Fig. 2. $[AP5]_o$ blockade of NMDAR fEPSP. Single experiment (5 stimuli, 100 Hz) demonstrating increased blockade of the synaptic response by elevating $[AP5]_o$. Under high pressure conditions (10.1 MPa) more than double $[AP5]_o$ was needed to achieve ~80% block. At high pressure, 20 min washout of the $[AP5]_o$ resulted in almost full recovery. All AP5 experiments were carried out at $[Mg^{2+}]_o=0$ mM.

EXPERIMENTAL PROCEDURES

Brain slices preparation

Animal experiments were carried out in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and in accordance with guidelines laid down by the institute ethics committee for the care and use of animal for experimental work. The preparation of rat corticohippocampal slices is described in detail in Mor and Grossman (2006). Regular Ringer solution contained (in mM) 124 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. Solution was constantly bubbled with 95% O_2 and 5% CO_2 for a pH of 7.4.

Pressure and compression

The pressure chamber and helium compression protocol are described in detail in Mor and Grossman (2006). Samples were taken at control (0.1–0.3 MPa, when 0.2–0.3 MPa were more stable for further compression) and at 10.1 MPa. At this stage, only one pressure step was used to reduce waiting time for equilibration and minimize rundown of the preparation. The single step to 10.1 MPa was chosen in order to obtain full expression of the HPNS since tremor and convulsions thresholds for rats are at 6 MPa and at 9 MPa respectively (Mor and Grossman, 2006; Brauer et al., 1974). Decompression was always attempted with good recovery at 0.2–0.4 MPa but only 20% success upon decompression to 0.1 MPa. Fresh Ringer solution containing no $[Mg^{2+}]_o$ at 30 °C (gas saturated at normobaric pressure, as above) was continuously injected into the experimental bath by a fast high-pressure pump. Consequently, the brain slices were washed with solutions maintaining constant levels of pH, antagonists' concentrations, and partial pressures of oxygen and CO_2 under control and hyperbaric conditions. Despite the fast wash of the solutions, only the partial pressure of helium in the tissue might have slightly increased, but even at the maximal compression (10.1 MPa) helium is considered to be an inert gas (see also Mor and Grossman, 2006, 2007).

Blockers application

In order to isolate the NMDAR response, AMPA receptors component of the fEPSP was blocked by the selective non-NMDAR antagonist 6,7–dinitroquinoxaline-2,3-dione (DNQX, 20 μM, Tocris, Bristol, UK). GABAergic inhibition was eliminated by picrotoxin (50 μM, Tocris, Bristol, UK). These blockers concentrations were maintained constant throughout the experiment (for details see Mor and Grossman, 2006). Mg^{2+} (as $MgCl_2$ salt) or DL-AP5 (Tocris, Bristol, UK) were added to the solutions in increasing concentrations up to the level of at least 75% NMDAR response block-

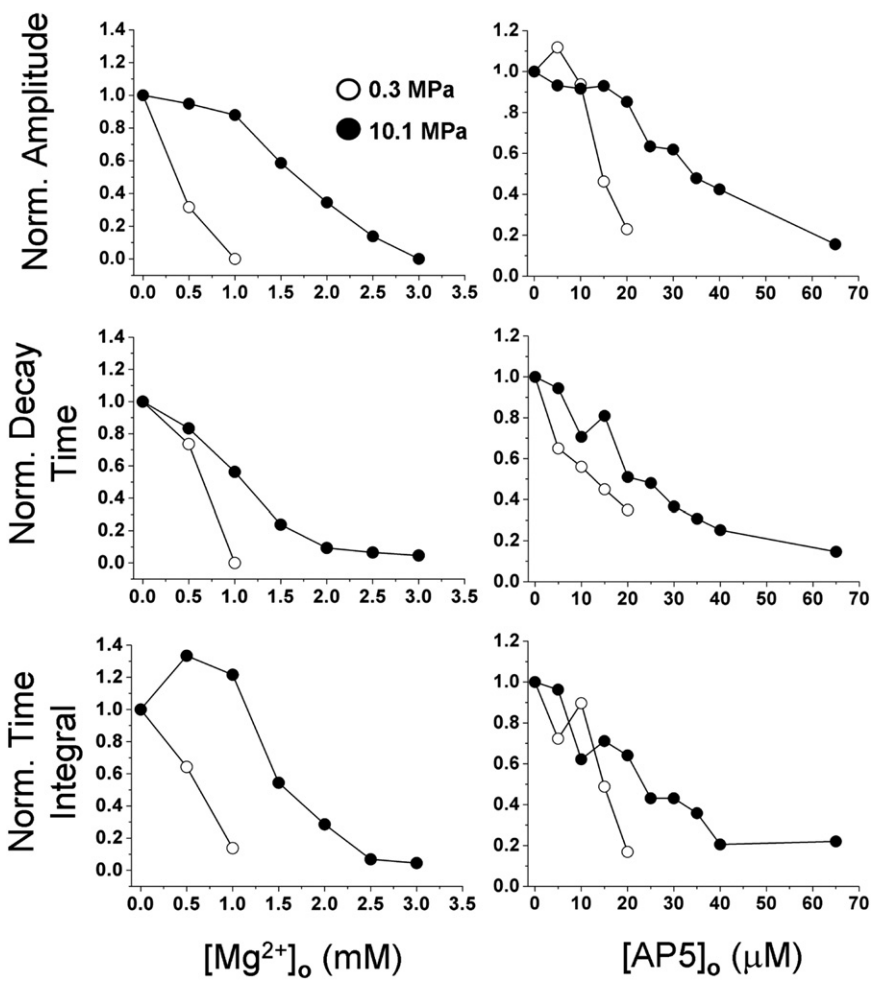

Fig. 3. High pressure shifts $[Mg^{2+}]_o$ and $[AP5]_o$ concentration-response curves to the right. The normalized concentration-response curves (example of single experiment for each blocker) demonstrate that higher $[Mg^{2+}]_o$ and $[AP5]_o$ were needed to decrease the NMDAR synaptic response's amplitude, decay time, and time integral at high pressure (a "shift to the right").

ade. The same protocol was applied for each slice preparation under control and hyperbaric conditions.

Stimulation and recording

Stimulation (250 μs duration) at submaximal intensities (4–6 mA) was conducted by bipolar tungsten microelectrode located on the hippocampus Schaffer collaterals pathway in CA2 region. Single isolated NMDAR synaptic response amplitude was usually low. Therefore, trains of three to five stimuli (25–100 Hz) were used in order to potentiate the responses (see also Mor and Grossman, 2006). Recording of evoked fEPSPs was performed by extracellular glass microelectrode (0.6–1.0 MΩ resistance) using Axopatch-1D amplifier (Molecular Devices, Axon Instruments, Inc., CA, USA) from the hippocampal CA1 pyramidal neurons at the apical dendritic layers. The presented responses are an average of five traces.

Data analysis

Synaptic recordings of CA1 fEPSPs at the apical dendritic layer of the hippocampus, their parameters, and their interpretation, were described in our previous publication (Mor and Grossman, 2006). Briefly, fEPSP amplitude, decay time and time integral were recorded in order to thoroughly assess the NMDAR response's magnitude. Time integral was calculated in order to provide an estimate for the cations influx magnitude (see also Mor and Grossman, 2006). Effective inhibitory concentrations (IC_{50}) were calculated and analysed for $[Mg^{2+}]_o$ and $[AP5]_o$. The experiments' results are expressed as mean$\pm$1 standard error of mean (SEM). Since high-pressure experiments often took a long time, eventually they were carried out using a single slice from each animal. The *n* expresses the number of successful experiments (number of slices) for each experimental protocol.

Statistical analysis

In each experiment, control conditions (0.1–0.3 MPa) and hyperbaric conditions (10.1 MPa) were tested on the same brain slice. Thus, we used paired samples *t*-test analysis, assuming electrophysiological recordings meet the conditions of a normal distribution. Paired *t*-tests were applied for the following scaled variables: fEPSP amplitude, fEPSP decay time, and fEPSP time integral. The degree of significance was denoted by the values of *P*. Results are considered statistically different when $P<0.05$. All statistical data were analyzed and calculated using SPSS13.0 (SPSS Inc., Chicago, IL, USA). Graphical representations were made by using Microsoft Office Excel (Microsoft Inc., Redmond, WA, USA) and SPSS 13.0.

RESULTS

High pressure reduced $[Mg^{2+}]_o$ and $[AP5]_o$ potency of blocking NMDAR responses

Under control conditions responses were blocked by the antagonists $[Mg^{2+}]_o$ or $[AP5]_o$ in a concentration-response manner. After reaching a significant blockade of $\geq$75% the antagonist was washed out and the responses recovered. Then, the same preparation was further compressed to 10.1 MPa. The responses were recorded again without the blocker and with increasing antagonist concentrations. Double to almost triple $[Mg^{2+}]_o$ (Fig. 1) and $[AP5]_o$ (Fig. 2) were needed to reach a near complete block of NMDAR responses.

High pressure shifts $[Mg^{2+}]_o$ and $[AP5]_o$ concentration-response curves to the right

The NMDAR fEPSPs' amplitude, decay time, and time integral, were significantly increased at high pressure (Mor and Grossman, 2006, 2007). These parameters are characteristic of the fEPSP capability to generate action potentials, and therefore were analyzed in detail. The concentration response (normalized to the "zero blocker" response) curves at pressure were all shifted to the right indicating a reduction in blockade efficacy of $[Mg^{2+}]_o$ and $[AP5]_o$ (Fig. 3).

Further statistical analysis (paired *t*-test) of $[Mg^{2+}]_o$ responses revealed that high pressure significantly increased IC_{50} for amplitude, decay time, and time integral by 210.0%$\pm$86.7%, 187.2%$\pm$59.4%, and 164.1$\pm$51.9% respectively (Fig. 4). The same analysis of $[AP5]_o$ responses showed that pressure similarly increased IC_{50} for amplitude, decay time, and time integral by 196.5%$\pm$58.7%, 219.0%$\pm$80.1%, and 201.4$\pm$85.8% respectively (Fig. 4, see also for *P*-values).

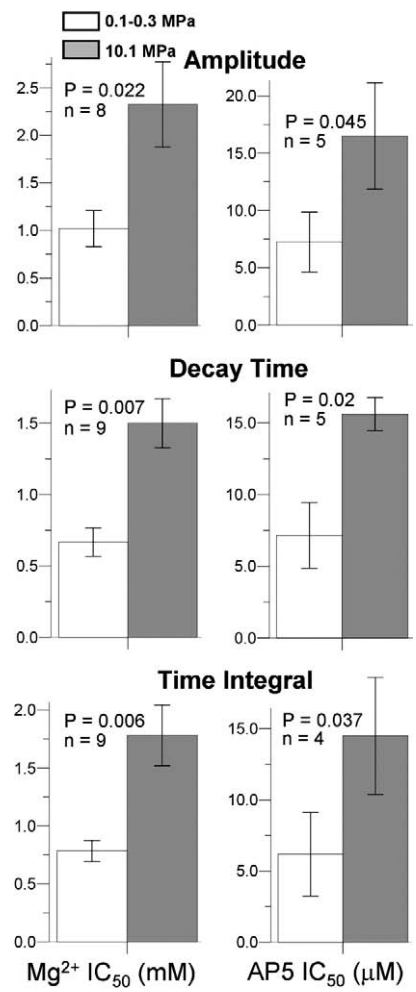

Fig. 4. Effects of high pressure on the $[Mg^{2+}]_o$ and $[AP5]_o$ IC_{50}. High pressure (10.1 MPa) significantly increased the $[Mg^{2+}]_o$ and $[AP5]_o$ required for 50% block of the NMDAR synaptic responses amplitude, decay time, and time integral. Statistical analyses: paired *t*-tests, *n*—number of experiments. Bars represent $\pm$1 SEM.

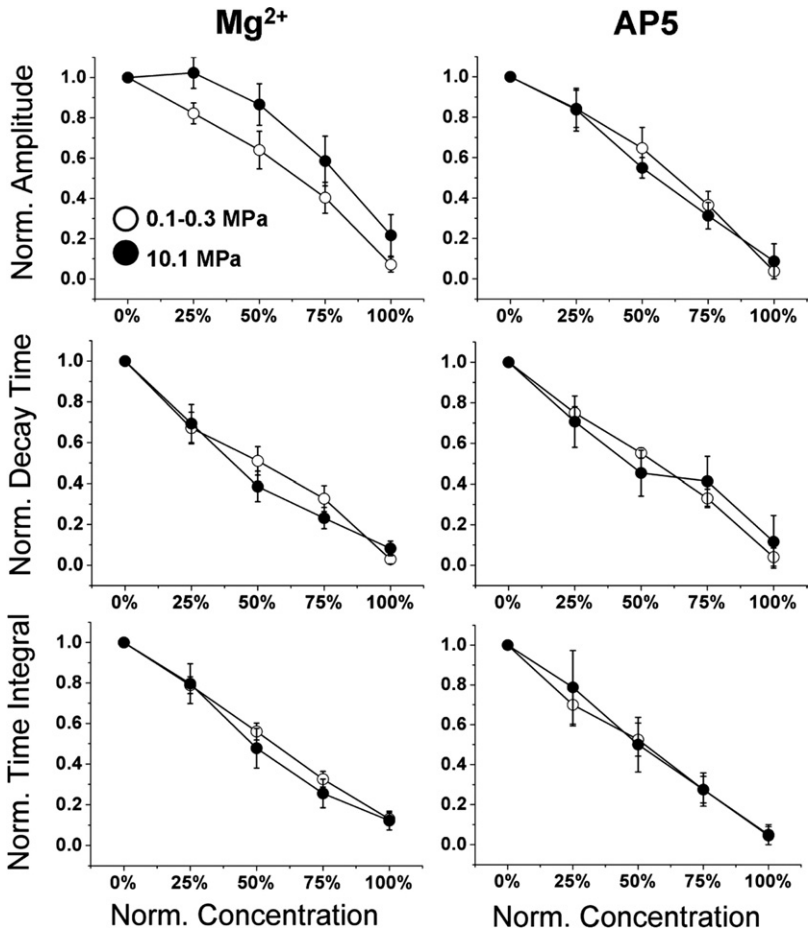

Fig. 5. High pressure does not change [Mg2+]o and [AP5]o blockade pattern of NMDAR response. Summarized normalized results for NMDAR synaptic responses' amplitude, decay time, and time integral vs. normalized [Mg2+]o and [AP5]o. Bars represent ±1 SEM. The number of experiments is identical to the values ("*n*") depicted in Fig. 4.

The pattern of $[Mg^{2+}]_o$ and $[AP5]_o$ blockade under normal and high pressure conditions

In order to compare the relative efficacy of the blockers at normal and high pressure, we normalized the blockers concentrations (0% represents no blockers in the solution, 100% represents maximal blocker effect) in addition to the normalization of the responses (as in Fig. 3). High pressure did not change the "double normalized" concentration-response curves of the three analyzed parameters. In other words, higher antagonists concentrations are needed to block NMDAR responses at high pressure but it seems that the pattern of the blockade is unchanged (Fig. 5, see discussion).

DISCUSSION

NMDAR is regulated by a variety of extracellular factors such as H^+, Mg^{2+}, Zn^{2+}, reductive or oxidative environment, and partial agonists and antagonists (Low et al., 2003; Williams et al., 1996; Choi and Lipton, 2000). NMDAR is also modulated by intracellular factors such as Na^+, phosphorylation (kinases), dephosphorylation (phosphatases) processes, and scaffolding proteins (Yamakura and Shimoji, 1999; Yu and Salter, 1998; Wenthold et al., 2003). As such, the receptor is involved in various cognitive, learning, and memory processes (Rebola et al., 2009). Deciphering the mode of action of $[Mg^{2+}]_o$ and $[AP5]_o$ on this receptor at high pressure may contribute significantly to understanding the involvement of NMDAR in short and possibly long term manifestations of HPNS and under other physiological or pathophysiological conditions (Marino and Conn, 2002; Petrenko et al., 2003; Rogawski and Wenk, 2003; Smith, 2003). The results show that $[Mg^{2+}]_o$ and $[AP5]_o$ blockade is significantly reduced at hyperbaric environment. Moreover, the physiological $[Mg^{2+}]_o$ blockade is practically abolished: double to triple the physiological $[Mg^{2+}]_o$ (1–1.5 mM) are needed to reach the same inhibitory effect at pressure. These functional changes suggest another mechanism for pressure augmentation of NMDAR response, in addition to those previously described (Mor and Grossman, 2006) including increased initial slope, larger amplitude and longer decay time of single NMDAR fEPSP under $[Mg^{2+}]_o=0$ conditions (i.e. in the absence of Mg^{2+} block).

Mg^{2+} occupies the channel pore and can be removed by membrane depolarization. However, Mg^{2+} blockade

elimination by simple pressure dependent membrane depolarization can be ruled out since no significant change in membrane potential and input resistance was reported for the CA1 pyramidal cells (identical to our preparation) recorded somatically at pressure (Southan and Wann, 1996). The practical elimination of Mg^{2+} blockade indicates that at pressure depolarization of the receptor by adjacent AMPA receptors or by any other input is no longer necessary for receptor activation. Thus, even under reduced synaptic glutamate release (see Introduction) the NMDAR can still be activated and even over activated.

Pressure induced shift to the right, in the concentration-response curves, seems to be similar for $[AP5]_o$ and $[Mg^{2+}]_o$ (Fig. 3). At least for AP5, that interferes with the glutamate binding site (see Introduction), it may indicate a "competitive inhibition." The general increase in extracellular (extra synaptic) concentration of glutamate reported for rat striatum (see Introduction) could barely support such a simple interaction. In contrast, the numerous reports about reduced evoked synaptic release imply lower concentration of glutamate at the synaptic cleft per se, which cannot support such a simple explanation. Furthermore, additional analysis (Fig. 5) suggests that pressure did not affect the mode of blockade but just shifted the range of concentrations for both antagonists. The simple assumption for the pressure effect(s) would be a conformational change in the receptor protein. However, conventionally such a change requires very high pressure beyond the range of pressures used in the present study (for review: Daniels and Grossman, 2003). It is difficult to assume a common pathway for pressure effects on both blockers (since they interact with different sites of the protein) unless we postulate a general modification in the protein (receptor)—phospholipids (membrane bilayer) interactions that may take place at lower pressure. Another "common effect" could be postulated based on recently suggested theory of protein's hydrophobic cavities. Schiltz et al. (2003) have used compressed xenon and krypton as heavy atoms in order to determine protein structure and Colloc'h et al. (2007) have studied protein crystallography under xenon and nitrous oxide pressure. These studies may suggest a new approach to the mechanisms of narcosis. However, if pressure per se could affect these "flexible hydrophobic cavities", it may lead to slower kinetics, and even conformational rigidity that will reduce accessibility of ions (and molecules such as our tested blockers) thereby interfering with the normal protein and cell function.

At this stage, we are unable to determine precisely the nature of any of the possible modifications. More sophisticated methods such as high pressure crystallography are necessary to decipher this question.

Nevertheless, this study provides further evidence and additional mechanism for pressure augmentation of NMDAR response. This augmentation results in enhanced fEPSPs involved in short term HPNS, and excessive Na^+ and Ca^{2+} influxes that may lead to long term excitotoxic neuronal injury in the CNS (Lynch and Guttmann, 2002).

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